PAX3

Gene Function

Paired box gene 3 (PAX3) is one of nine PAX genes, all of which encode a protein containing a highly conserved, 128 amino acid paired-box DNA-binding domain, named based on its similarity to the DNA-binding domain of the Drosophila paired protein (Chi and Epstein, 2002). **PAX3, -4, -6, and -7 each contain an additional DNA-binding domain, the paired-type homeodomain.**

The binding site recognized by PAX3 contains an ATTA motif, bound by the homeodomain, and a GTTCC motif bound by the paired domain. While the PAX3 paired domain can bind independently, **the homeodomain requires the presence of the paired domain to bind DNA**. The region between the two DNA-binding domains contains a conserved octapeptide that functions in homodimerization (Chalepakis et al., 1994b).

PAX3 can activate or inhibit transcription. Transient transfections of a variety of PAX3 deletion constructs revealed that PAX3 appears to act as a transcriptional activator over a narrow concentration range, as high concentrations of PAX3 showed very little transcription activation (ie. inhibition). The authors suggest that the phenotypes of WS patients and splotch mice harboring heterozygous PAX3 mutations could result from this apparent concentration-dependence of PAX3 function, altering cell fate because reduced PAX3 concentrations elicit different transcriptional programs. Also, **the N-terminus of PAX3 contains a transcription inhibition domain, while the C-terminus contains a transcription activation domain** (Chalepakis et al., 1994b).

Numerous studies have analyzed the function of the two DNA binding domains of PAX3, demonstrating **that the PAX3 homeodomain and paired domain interact to regulate DNA binding**. This interaction of the domains was originally identified from analysis of the Splotch delayed (Sp^d) allele, which encodes a full-length *Pax3* mRNA containing a point mutation within the paired domain. Oligo binding analysis showed that the DNA-binding ability of both the homeodomain and the paired domain was reduced in the Sp^d protein, even though only the paired domain was mutated (Underhill et al., 1995). **Further analysis implicated the beta-hairpin motif within the paired domain as crucial for DNA binding by both the homeodomain and the paired domain**. Also, the paired domain appears to limit the sequence recognition of the homeodomain, as wild type PAX3 does not bind to synthetic oligonucleotides containing homeodomain recognition motifs separated by a 3-base pair spacer (P3), while deletion of the paired domain of PAX3 allowed binding to these P3 sequences (Underhill and Gros, 1997). Analysis of PAX3 mutations found in WS patients showed that seven paired domain mutations (all within the N-terminal region of the paired domain) and one homeodomain mutation affected the binding of both DNA-binding domains. Structure prediction for DNA-bound PAX3 showed that many of these mutated residues clustered near the minor groove and phosphate backbone of DNA (Fortin et al., 1997). Mutagenesis studies showed that mutation of the homeodomain affected the DNA-binding ability of the paired domain, and vice-versa (Apuzzo and Gros, 2002). Further analysis using protease sensitivity studies showed that DNA-bound PAX3 adopts a more compact structure, and binding of the homeodomain results in conformational changes in the paired domain, and vice versa (Apuzzo et al., 2004). Biochemical cross-linking analyses suggested that the PAX3 tertiary structure brings the second helix of the paired domain in close proximity with the N-terminus of the homeodomain (Apuzzo and Gros, 2007).

A variety of mutagenized *Pax3* and *Pax3-Fkhr* constructs also showed that the **transactivation domain influences the DNA binding properties of PAX3** (Cao and Wang, 2000).

The crystal structure of wild type PAX3 homodimer bound to DNA was determined, illustrating how many of the PAX3 missense mutations associated with WS1/3 disrupt normal DNA binding by the PAX3 homeodomain. The PAX3 homeodomain exhibits the canonical fold demonstrated by other homeodomain structures, with the recognition helix binding the major groove, and the N-terminal portion fitting into the

minor groove and aiding in DNA binding and homodimerization. PAX3 binding induces a very moderate 3° bend in DNA, which is smaller than that induced by any other homeodomain structures to date. Numerous water molecules are located between the PAX3 homeodomain and DNA, suggesting that the specific binding would be able to accommodate significant DNA conformational changes (Birrane et al., 2009).

***Pax3* is mutated in the classic mouse mutant Splotch (*Sp*)**, which displays white belly spotting, and occasional white spotting seen on the back, feet and tail in heterozygotes (Russell and Roscoe, 1947). Homozygotes exhibit embryonic lethality, displaying spina bifida (rachischisis) and kinky tail, exencephaly, craniofacial abnormalities, persistent truncus arteriosis, and skeletal muscle defects. A number of Sp alleles exist, from spontaneous ($Pax3^{Sp\text{-}d}$) (Dickie, 1964) and X-irradiation induced ($Pax3^{Sp\text{-}1H}$ and $Pax3^{Sp\text{-}2H}$, $Pax3^{Sp\text{-}r}$) (Beechey and Searle, 1986), to more recent alleles generated by chemical mutagenesis ($Pax3^{Sp\text{-}6H}$, $Pax3^{Sp\text{-}7H}$) (Bogani et al., 2004).

Detailed analysis of *Sp* homozygotes reported phenotypes first visible at E9.5, including open neural folds in the head and lumbo-sacral region, and neural and cranial tissue overgrowth, reduced dorsal root and sympathetic ganglia, and abnormal tail formation. Pigmentation in the retinal pigmented epithelium is normal in *Sp/Sp* embryos. Transplantation experiments demonstrated that *Sp/Sp* integument transplanted into chick coelum was not capable of forming pigment (Auerbach, 1954).

The first identification of *Pax3* as the gene mutated in *Sp* was directed by the similar map location of *Pax3* and *Sp* and the overlap of embryonic *Pax3* expression with the tissues affected in *Splotch* mice. **Candidate gene analysis of *Pax3* in Sp^{2H} mice revealed that Sp^{2H} mice harbor a 32 nucleotide deletion that contains part of the homeodomain** (Epstein et al., 1991b).

The developmental defects of neural crest in *Pax3* mutant mice were assayed by crossing $Pax3^{Sp1H}$ mice with mice carrying the HCMV-IEP-lacZ (CMZ) transgene, which is expressed in apical neural folds, premigratory neural crest, and spinal and cranial ganglia. The results demonstrated that **$Pax3^{Sp1H}$ mice exhibit defects in spinal and sympathetic ganglia formation** (Franz and Kothary, 1993).

Similar *Pax3* mutations in mouse and man resulted in similar phenotypes, and the differences between phenotypes of Splotch mouse mutants and Waardenburg syndrome (WS) individuals are the result of genetic background variation (Tassabehji et al., 1994).

Specific upregulation of the chondroitin sulfate proteoglycan *versican (Vcan)* mRNA was seen in the tissue surrounding the paths of normal neural crest migration in homozygous E9.5-E11.5 $Pax3^{Sp2H}$ embryos. Detailed in situ analysis showed that the locations of increased *Vcan* expression correlated with the paths of missing neural crest cells in both the mesenchyme lateral to the neural tube and in branchial arches 4 and 6. Because *Pax3* and *Vcan* are expressed in mutually exclusive patterns during development of wild type embryos, the authors hypothesized that normally PAX3 functions to inhibit *Vcan* expression, as VCAN inhibits neural crest cell migration (Henderson et al., 1997).

Pax3 transgenic mice were generated using the proximal 1.6kb promoter region upstream of *Pax3* and full-length *Pax3*. Transient transfections with a *LacZ* reporter construct demonstrated that the 1.6kb construct directed expression of *Pax3* in neural tube and neural crest, but not somites. Crossing of *Pax3* transgenics onto a homozygous Splotch background showed rescue of neural crest–related abnormalities–neural tube development, cardiac development, pigmentation, and facial bones and tongue–while muscular abnormalities of the trunk and limbs persisted. Therefore **the 1.6kb promoter region is necessary and sufficient to direct *Pax3* expression in neural tube and developing heart. They also demonstrated cell-autonomous functions of *Pax3* in neural crest, and showed that Pax3 expression in developing somites does not cause neural crest defects due to non-cell autonomous effects on migration**. *Pax3* transgenics did not show any tumors or other abnormalities (Li et al., 1999).

Construction of transgenic mice expressing -galactosidase under control of the melanogenic enzyme *Dct (Dct-LacZ)* allowed lineage tracing of melanoblasts in vivo in the context of mutant *Pax3*.

***Dct-LacZ*-expressing melanoblasts in *Pax3* homozygous mutant embryos were greatly reduced in number, but still showed normal migration, suggesting PAX3 regulates development of melanoblasts or melanoblast precursors rather than later stages of melanoblast migration/differentiation**. This is contrasted with the complete absence of *Dct-LacZ*-expressing melanoblasts in *Mitf* mutant embryos, demonstrating MITF is required for melanoblast survival (Hornyak et al., 2001).

Chimeric mice were generated using mice carrying $Pax3^{2H}$ alleles as well as a $Pax3^{LacZ}$ targeted gene knock-out ($Pax3^{2G}$), so that chimeric mice had subsets of easily identifiable $Pax3^{-/-}$ cells in the context of surrounding wild type cells. Analyses indicated that **neural crest cells that are deficient for *Pax3* can still migrate properly**, as shown by normal migration along dorso-lateral and dorso-ventral pathways in grafts of $Pax3^{+/-}$ and $Pax3^{-/-}$ tissue into chick embryos. In these chick grafts, a higher percentage of $Pax3^{-/-}$ cells migrated along the dorso-lateral pathway. In the neural tube and dermomyotome, $Pax3^{-/-}$ cells contributed to the developing neural tube and dermomyotome, but did not intermingle with wild type cells, and both neural tube and dermomyotome exhibited defects reminiscent of Splotch homozygote embryos. Taken together, the results indicated that *Pax3* acts in the neural tube and somites in a cell-autonomous fashion, potentially by controlling cell surface properties, but does not regulate neural crest migration cell-autonomously (Mansouri et al., 2001).

Previous analyses of $Pax3^{Sp/Sp}$ embryos found that neuroepithelial apoptosis was seen at sites of neural tube defects (Phelan et al., 1997). Crosses of $Pax3^{Sp/+}$ mice with mice heterozygous for a knockout of the tumor suppressor p53 showed that **absence of functional p53 rescues the neural tube defects and cell death in $Pax3^{Sp/Sp}$ embryos**. Administration of the p53 inhibitor pifithrin-alpha replicated this effect. Quantitative analysis of E10.5 embryos showed that *Pax3* mutations result in an increase in p53 protein, but not mRNA, in embryos. These results suggest PAX3 does not specify a neural crest developmental program, but instead regulates a p53-dependent pathway to maintain the neural crest by preventing cell death (Pani et al., 2002).

Ectopic PAX3 expression in pRb-negative human osteosarcoma and rhabdomyosarcoma cell lines triggered the formation of cellular aggregates and a mesenchymal to epithelial transition. Cell shape and membrane and cytoskeletal architecture were altered, cell adhesion was increased, and epithelial-like cell-cell contacts were formed. However, PAX3 expression also altered cell conditions (via *c-met* activation) to allow these cells to be responsive to hepatocyte growth factor/scatter factor (HGF/SF), which subsequently caused an epithelial to mesenchymal transition. The authors suggest that the absence of PAX3 removes regulation of cell adhesion and epithelial cell morphogenesis, thus mechanistically explaining the neural tube defects and dermomyotome abnormalities in *Pax3* mutant mice (Wiggan et al., 2002).

Further analysis of the ability of PAX3 to induce cellular aggregate formation identified structural and molecular changes caused by PAX3 overexpression. PAX3 overexpression induces cells to form highly extended three-dimensional processes, causes re-localization of Disheveled and Frizzled to the actin cytoskeleton near the cell membrane, and causes activation and relocation of JNK to juxta-nuclear vesicular structures (Wiggan and Hamel, 2002).

Immunohistochemistry using mice in which endogenous *Pax3* was replaced by Cre under control of the *Pax3* promoter demonstrated that the *Pax3* promoter directed Cre expression in putative melanocyte stem cells, within the bulge region of the hair follicle. A variety of binding assays showed that at similar concentrations, PAX3 activates *Mitf*, thus stimulating a program for melanogenesis, but also functions as an inhibitor of Dct by competing with MITF at an enhancer of *Dct* and recruiting the Groucho repressor GRG4, thus limiting differentiation by blocking MITF activation of *Dct*. When activated beta-catenin is expressed, it displaces PAX3 (and GRG4) from the *Dct* promoter, allowing MITF to activate *Dct* expression. These data suggest that **PAX3 functions at a nodal point in adult melanocyte stem cell differentiation, allowing melanocyte stem cells to remain undifferentiated via *Dct* inhibition, yet committed to the melanocyte lineage by activating *Mitf***. In this model, the stem cells accumulate a high concentration of MITF so that upon external stimulation, such as sun exposure, the activation of beta-catenin would remove PAX3 inhibition of *Dct*, and allow the cells to rapidly activate downstream melanogenic genes (Lang et al., 2005).

A variety of assays performed on stably transfected mouse melan-a cells revealed that **the 7 PAX3 isoforms have different effects on cell growth, survival, migration, and transformation**. Both PAX3a and PAX3b inhibited proliferation; PAX3b also accelerated apoptosis and reduced migration. Both PAX3c and PAX3d promoted growth, migration, transformation, and survival. PAX3e reduced growth and increased apoptosis. PAX3g reduced migration, and PAX3h increased proliferation, migration, survival, and transformation (Wang et al., 2006).

Transient transfections using promoter constructs that are activated by p53 showed that **PAX3 can repress p53-induced transcription**; this repression was seen in two out of three promoters (*HDM2-P2* and *BAX*, but not *WAF1*). PAX3 reduced p53 protein levels, however this reduction is not regulated through p53 interaction with its cellular inhibitor, HDM2, the most commonly known mechanism for p53 protein degradation. A variety of *PAX3* mutant constructs demonstrated that the **PAX3-mediated repression of p53 requires PAX3 to have intact transcriptional activation and DNA binding domains**. The authors suggest that PAX3 may regulate p53 through either an alternative ubiquitin E3-ligase or an ubiquitin-proteosome-independent pathway (Underwood et al., 2007).

Fortuitous creation of a hypomorphic *Pax3* allele (*$Pax3^{neo}$*), exhibiting 80% reduction in expression and postnatal lethality prior to weaning in homozygotes, showed that PAX3-expressing tissues show variable sensitivity to reduced PAX3 levels. The long range migration of myoblasts into the limb and tongue showed the greatest defects caused by apoptotic cell death, while neural tube closure, neural crest development, and other myoblast development was normal. PAX7 expression was upregulated in neural tube and somites (in contrast to reduced PAX7 expression in *$Pax3^{Sp2H}$* null embryos), suggesting PAX7 compensates for PAX3 in neural tube, neural crest, and myoblast migration to trunk, body wall, intercostal space, and diaphragm when *Pax3* levels are reduced. Heterozygous *$Pax3^{neo/+}$* mice showed no belly spots, suggesting melanocytes are not sensitive to the approximately 60% reduction in PAX3 expression seen in these mice (Zhou et al., 2008).

Mice carrying a Cre-inducible Pax3 construct where PAX3 expression persisted in cranial neural crest showed craniofacial bone malformations, including ocular defects and cleft palate that resulted in perinatal lethality. This persistent PAX3 expression caused the developing neural crest to be unresponsive to bone morphogenic protein signaling, **suggesting that a general mechanism of PAX3 downregulation allows differentiation factor responsiveness in neural crest** (Wu et al., 2008).

Because *PAX3* mutations in WS1 and WS3 individuals show variable phenotype-genotype correlations, and because the molecular basis of *PAX3* mutations encoding loss-of-function protein alterations are unclear, the subnuclear localization and mobility of PAX3 proteins harboring missense mutations were analyzed. The results indicated that **there are two classes of DNA-binding domain PAX3 missense mutations: Class 1 PAX3 mutants showed greatly increased mobility as well as diffuse nuclear localization, while Class 2 PAX3 mutants had moderately increased mobility but retained normal localization**. Interestingly, these mobility increases were inversely correlated with DNA binding, as the Class 1 mutants with greatest mobility retained DNA binding. The Class 1 and Class 2 mutations did not segregate by the location of mutations within the PAX3 protein; rather, it appears that the more severe Class 1 mutations completely disrupt important tertiary or quaternary structures, while Class 2 mutations retain structural interactions that regulate localization. **These results indicate that altered protein mobility is key in mediating PAX3 mutant phenotypes, regardless of the ability of the mutant protein to bind DNA or activate reporter genes** (Corry et al., 2008).